



Winters, A. E., Wilson, N. G., van den Berg, C. P., How, M. J., Endler, J. A., Marshall, N. J., White, A. M., Garson, M. J., & Cheney, K. L. (2018). Toxicity and taste: unequal chemical defences in a mimicry ring. *Proceedings of the Royal Society B: Biological Sciences*, 285(1880), [20180457]. <https://doi.org/10.1098/rspb.2018.0457>

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Link to published version (if available):  
[10.1098/rspb.2018.0457](https://doi.org/10.1098/rspb.2018.0457)

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## **Toxicity and taste: unequal chemical defences in a mimicry ring**

Journal:	<i>Proceedings B</i>
Manuscript ID	RSPB-2018-0457
Article Type:	Research
Date Submitted by the Author:	28-Feb-2018
Complete List of Authors:	Winters, Anne; The University of Queensland, School of Biological Sciences Wilson, Nerida; Western Australian Museum, van den Berg, Cedric; University of Queensland, School of Biological Sciences How, Martin; The University of Bristol, School of Biological Sciences Endler, John; Deakin University, Life & Environmental Sciences; James Cook University, Marine & Tropical Biology Marshall, Justin; University of Queensland, School of Biomedical Sciences White, Andrew; University of Queensland Garson, Mary; University of Queensland, School of Chemistry & Molecular Biosciences Cheney, Karen; University of Queensland, School of Integrative Biology
Subject:	Evolution < BIOLOGY, Ecology < BIOLOGY, Behaviour < BIOLOGY
Keywords:	mimicry, chemical defences, aposematism, invertebrates, visual ecology
Proceedings B category:	Evolution

1    **Toxicity and taste: unequal chemical defences in a mimicry ring**

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**Abstract**

Mimicry of warning signals is common, and can be mutualistic when mimetic species harbour equal levels of defence (Müllerian), or parasitic when mimics are undefended but still gain protection from their resemblance to the model (Batesian). However, whether chemically defended mimics should be similar in terms of toxicity (i.e. causing damage to the consumer) and/or unpalatability (i.e. distasteful to consumer) is unclear and in many studies remains undifferentiated. In this study, we investigated the evolution of visual signals and chemical defences in a putative mimicry ring of nudibranch molluscs. First, we demonstrated that the appearance of a group of red spotted nudibranchs molluscs was similar from the perspective of potential fish predators using visual modelling and pattern analysis. Second, using phylogenetic reconstruction, we demonstrated that this colour pattern has evolved multiple times in distantly related individuals. Third, we showed that these nudibranchs contained different chemical profiles used for defensive purposes. Finally, we demonstrated that although levels of distastefulness remained relatively constant between species, toxicity levels varied significantly. We highlight the need to disentangle toxicity and taste when considering chemical defences in aposematic and mimetic species, and discuss the implications for aposematic and mimicry signal evolution.

**Key words:** mimicry rings, chemical defences, aposematism, marine invertebrates, nudibranch

## 39 **Introduction**

40 Many animals use visual displays to advertise they are chemically or otherwise  
41 defended (aposematism) [1]. The efficacy of aposematic signals in deterring predation is  
42 thought to be frequency-dependent, as the warning signal must be encountered multiple times  
43 for predators to learn and remember the association between the signal and level of  
44 unpalatability [2-5]. Müllerian mimics are defended species that have a mutualistic  
45 relationship with co-mimics to increase encounters with predators and spread the burden of  
46 predator learning [6-8], whereas Batesian mimics are undefended species that parasitize the  
47 warning signal of their defended sympatric model [9]. However, mimicry systems are  
48 thought to lie on a spectrum of chemical defence strength, with well-protected Müllerian  
49 mimics at one end, unprotected Batesian mimics on the other, and a range of intermediate  
50 protection in between (quasi-Batesian mimics) [5, 10-14].

51 When investigating the relative strength of chemical defences for species in proposed  
52 mimicry rings, studies tend to consider the unpalatability of species (i.e. distastefulness to  
53 consumer) [15-18] and/or toxicity (i.e. harm to consumer) [19-21]. However, the relationship  
54 between distastefulness and toxicity in chemically defended prey is rarely investigated and  
55 surprisingly, in many studies remains undifferentiated (but see [22]). Indeed, distastefulness  
56 and toxicity are often used synonymously in the literature [5, 19, 23], with perhaps the  
57 assumption that they are correlated. Prey species that are distasteful but not toxic, or vice  
58 versa, may be common [5, 24], and therefore the relationship between distastefulness and  
59 toxic defence needs further consideration [25]. Distasteful compounds that are non-toxic  
60 could initially deter predators [26], but may eventually be accepted by predators [26, 27].  
61 This could be dependent on predator satiation, how unpleasant the compound is and the  
62 abundance of other palatable prey items [28]. Therefore, toxicity could be considered a more

effective deterrent than distastefulness. However, distasteful compounds that are moderately toxic may also protect prey populations more effectively than highly toxic compounds [29].

To investigate the relationship between distastefulness and toxicity in mimicry systems, we investigated a putative red spot mimicry ring of nudibranch molluscs that co-occur along the east coast of Australia [30, 31]. Many species of nudibranchs display vibrant warning colours to indicate that they contain defensive secondary metabolites that are sequestered, transformed from dietary sources, or synthesized *de novo* [32]. We have previously shown that one member of the putative red spot mimicry group, *Goniobranchus splendidus*, contains distasteful compounds to marine organisms and displays conspicuous colours patterns, components of which are learnt readily by reef fish predators [33]. We first examined the similarity of colour patterns in this group to a potential fish predator using spectral reflectance measurements, visual modelling and pattern geometry analysis. Second, we conducted phylogenetic analysis to investigate shared ancestry of species. Third, we identified and quantified defensive metabolites present in each species and examined the strength of chemical defences using anti-feedant and toxicity assays with shrimp.

## Methods

### *Study species*

Nudibranch species ( $n = 24$ ) were collected between 2012 and 2016 by hand from sites in Queensland (QLD) and New South Wales (NSW) (Table S1) either on SCUBA at depths ranging from 5-18m, or from intertidal zones. Based on previous groupings [30], we identified species that exhibited a similar red spotted or red reticulate colour pattern and/or a distinctive yellow/orange mantle border (Figure 1, A-H). Eight species of nudibranch were assigned a priori to a red spot mimicry group: *Goniobranchus splendidus* (Angas, 1864) ( $n = 22$ ), *G. tinctorius* (Rüppell & Leuckart, 1830) ( $n = 4$ ), *G. daphne* (Angas, 1864) ( $n = 8$ ), *G.*

88 *hunterae* (Rudman, 1983) (n = 1), *Mexichromis mariei* (Crosse, 1872) (n = 4), *Mexichromis*  
 89 *festiva* (Angas, 1864) (n = 32), *Hypselodoris bennetti* (Angas, 1864) (n = 26), and *Verconia*  
 90 *haliclona* (Burn, 1957) (n = 1). We assigned a further four species to a partial red spot pattern  
 91 group: *G. verrieri* (Crosse, 1875) (n = 2), *G. albonares* (n = 5), *G. tasmaniensis* (Bergh,  
 92 1805) (n = 5), and *Chromodorididae thompsoni* (generic placement unassigned, Johnson &  
 93 Gosliner 2012) (n = 3). These species exhibit part of the red spot mimicry pattern, either with  
 94 spots or a coloured mantle border missing, or spots of a different colour (Figure 1, I-L).  
 95 These twelve species co-occur in the study area, and seven of these species are endemic [30].

96 A further 12 species were assigned to a non-mimic group: *Ceratosoma amoenum*  
 97 (Cheeseman, 1886) (n = 4), *Chromodoris kuiteri* Rudman, 1982 (n = 4), *C. lochi* Rudman,  
 98 1982 (n = 3), *C. elisabethina* (Bergh, 1877) (n=6), *Doriprismatica atromarginata* (Cuvier,  
 99 1804) (n = 4), *Goniobranchus decorus* (Pease, 1860) (n = 2), *G. geometricus* (Risbec, 1928)  
 100 (n = 2), *Hypselodoris jacksoni* Wilson and Willan 2007 (n = 2), *H. obscura* (Stimpson, 1855)  
 101 (n = 6), *H. tryoni* (Garrett, 1873) (n = 3), *H. whitei* (Adams and Reeve, 1850) (n = 3),  
 102 *Risbecia godeffroyana* (Bergh, 1877) (n = 2). These species do not appear to closely resemble  
 103 the red spot mimicry group in terms of colour combinations or pattern (Figure S1).

104 All specimens were placed in buckets with aerated seawater, transported to the  
 105 laboratory and placed in a petri dish of seawater for processing. The extended crawling length  
 106 (cm) of each individual was measured, individuals were photographed, the spectral  
 107 reflectance of each distinct colour pattern element was measured in the water with a  
 108 spectrophotometer, and a small portion of tissue from the tail was placed in ethanol for  
 109 phylogenetic analysis. Species identifications were confirmed through expert examination  
 110 (N.G.W) and genetic sequencing of Cytochrome *c* Oxidase I (COI) and 16S rDNA and  
 111 comparison with sequences deposited on the database GenBank. All nudibranch specimens  
 112 were then frozen and stored at -20°C until chemical extraction of chemical defences.

*Phylogenetic relatedness*

Representative individuals of newly-collected species selected for the phylogeny were extracted with a DNeasy blood and tissue kit (Qiagen). These were used in PCR reactions to amplify two mitochondrial genes, COI and 16S, using the primers and methods of Wilson, Maschek [34]. Details of all species used in the phylogenetic analysis are available in Table S2. All available COI and 16S data for the Chromodorididae was downloaded from GenBank and added to newly generated data from this study (COI GenBank XXXXX; 16S GenBank XXXXX). Only individuals that were represented by both genes from the same individual were used. This resulted in a data set with 146 species, representing an estimated 40% taxon completeness for the family ([www.marinespecies.org](http://www.marinespecies.org)). Data were aligned using the MAFFT v7.222 algorithm implemented in Geneious v 9.0.5, trimmed of primer regions, and checked for translation (COI). Data for each gene fragment were analysed separately in a maximum-likelihood (ML) framework for error checking and then concatenated but partitioned, applying the optimal models of evolution simultaneously estimated and selected with the Bayesian Information Criterion in ModelFinder [35] executed in IQ-TREE [36]. To estimate support at each node we used the ultrafast bootstrap function, implementing 1000 replicates using a maximum of 1000 iterations and a minimum correlation coefficient of 0.99 as a stopping rule [37]. Outgroups from the putative sister group Actinocyclusidae were added, as well as other members of the Dorididae, allowing for outgroup uncertainty recently highlighted [38]. The tree was rooted with *Doris kerguelensis*. We mapped ancestral traits of red spot mimic colour signals (0, no red spot pattern; 1, partial red spot pattern; 2, full red spot pattern) using stochastic character mapping (SCM) [39] in Mesquite v 3.2 [40]. We selected 'MK1' as the evolutionary model, which assumes an equal probability for a particular character change.



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139

140 *Spectral reflectance measurements*

141       Spectral reflectance measurements of each nudibranch colour pattern element were  
142 obtained by placing individuals in a dish immersed in seawater and measurements were taken  
143 with an Ocean Optics USB2000 spectrophotometer (Dunedin, FL, USA) and Ocean Optics  
144 OOIBASE32 software. We used a 200  $\mu\text{m}$  bifurcated optic UV/visible fibre held underwater  
145 at 45° angle connected to a PX-2 pulse xenon light (Ocean Optics). The percentage of light  
146 reflected at each wavelength from 300-700 nm was calibrated using a Spectralon 99% white  
147 reflectance standard (LabSphere, NH, USA) placed in the petri dish of seawater with the  
148 nudibranch. At least 10 measurements were taken of each colour pattern element and  
149 averaged per individual. Spectral reflectance data were not obtained for specimens of  
150 *Verconia haliclona* or Chromodorididae *thompsoni* due to equipment failure and therefore  
151 these species were not included in the colour pattern analysis.

152

153 *Colour and pattern analysis*

154       We first quantified colour pattern elements from the perspective of a potential  
155 trichromatic fish predator, the triggerfish *Rhinecanthus aculeatus* (photoreceptor  $\lambda_{\text{max}}$  of 413  
156 nm, 480 nm, 528 nm and transmission measurements through cornea, vitreous and lens, all as  
157 per [41]). We used this species to model the visual characteristics of nudibranchs as it is an  
158 omnivorous fish known to prey on molluscs, found throughout the range of the proposed red  
159 spot mimicry group (OZCAM.com.au) and is also representative of a common trichromatic  
160 visual system found in many marine fish species [42].

161       Photon capture generated by each given colour pattern element (i) for each  
162 photoreceptor  $q_i$  was calculated as per equation 1 in [43]. Irradiance measurements,  $I(\lambda)$ , were

163 taken at a depth of 5 m (as per [44]). Photon loss by transmittance in function of distance was  
164 ignored due to the relative clarity of the water in shallow reefs and the small distance  
165 assumed between object and viewer (max 30cm). In order to incorporate colour constancy,  
166 cone capture quanta were transformed using the von Kries correction as per equation 2 in  
167 [43].

168 Each colour pattern element was defined as an internal pattern (spots, stripes,  
169 reticulate), overall body (background) colour and, if present, a contrasting rim. Colour pattern  
170 elements were plotted in a trichromatic visual space (Maxwell's triangle) and we measured  
171 hue (the angle of the colour coordinate relative to the achromatic point), chroma (or  
172 saturation, defined as its distance from the achromatic point) and luminance (measured used  
173 the combined photon capture of the double cone, which process luminance in reef fish [45])  
174 from each colour pattern element. Methods were modified from [46, 47].

175 For pattern analysis, we used images of nudibranch that were normalized for size by  
176 rescaling the images to a standard body area of 5000 pixels. The outline of each animal was  
177 then manually traced using a magnetic lasso tool and extracted from the background using  
178 Adobe Photoshop CS5. The nudibranch image was then stylized for analysis by placing a  
179 transparent layer over the original image and using the pencil tool to define the red spot  
180 pattern [48]. This ensured individual colour pattern elements were correctly recognized by  
181 the MATLAB code required to run the analysis. Pattern properties of the entire nudibranch  
182 pattern were quantified using the adjacency analysis method [48]. Briefly, the method  
183 quantifies the distribution of transitions within and between colour pattern elements on an  
184 animal. Three relevant statistics [48] were calculated: 1) aspect ratio, 2) colour diversity and  
185 3) pattern complexity. Aspect ratio was calculated by dividing the vertical patch size by the  
186 horizontal patch size (patch size was determined by calculating the average number of pixels  
187 along a vertical or horizontal transect until a zone transition). Colour diversity described how

spatially evenly colours are represented in the pattern. High values indicate that the relative areas of each colour class are more close to being equal; diversity was calculated by the inverse Simpson index which yields the equivalent number of equally common (area) colours. Pattern complexity was calculated as the density of colour transitions; patterns with a greater number of pixels adjacent to a different colour class will have a higher complexity score.

#### *Non-metric multidimensional scaling analysis (NMDS)*

Species were differentiated in two-dimensional space using 14 characters of colour and pattern analysis by performing a non-metric multidimensional scaling analysis based on a Euclidean distance matrix with the metaMDS function in the vegan package [49] of R v 3.2.2 [50]. Characters were overall pattern (plain = 1, reticulate = 2, spotted = 3 or striped = 4); chromatically contrasting rim (absent = 0, present = 1); hue, chroma, luminance of internal pattern, background colour and rim; and our three pattern geometry statistics (aspect ratio, colour diversity, pattern complexity). If there was more than one pattern present on the species, then the dominant pattern was used as defined by 3 authors and is stated in Table S3. If internal patterns or rims were not present on a particular species, then values calculated for background colour were used.

#### *Chemical extraction and identification*

To investigate the identity and strength of chemical defences for each species, the whole body tissue of specimens were extracted as per [51]. All extracts were dissolved in deuterated chloroform for  $^1\text{H}$  NMR analysis on a Bruker AV-500 spectrometer at 500 MHz. If necessary for identification of nudibranch metabolites, a small portion of the extract was analyzed using low-resolution electrospray ionisation mass spectrometry (LRESIMS) on a Bruker Esquire HCT mass spectrometer. The  $^1\text{H}$  NMR and LRESIMS data of crude extracts

were compared with the respective literature to identify known compounds. Where necessary, a small portion of the extract was subjected to silica flash chromatography, and the various fractions produced were further separated into individual compounds by normal phase high performance liquid chromatography (NP HPLC), eluting with various ratios of hexanes/ethyl acetate. Dried extracts were placed in solution with dichloromethane (DCM) at the recorded specimen volume to provide a stock solution at the natural concentration (mg/mL) of extract for use in toxicity and palatability assays.

### *Toxicity Assay*

In order to measure the relative toxic properties of crude extracts from each species of nudibranch, brine shrimp (*Artemia* sp.) LD<sub>50</sub> (Lethal dose at 50%) assays were conducted between November 2013 and September 2015 on six of the twelve red spot species for which there was enough biological material (*G. splendidus*, *G. tinctorius*, *G. daphne*, *G. tasmaniensis*, *M. festiva*, *H. bennetti*). Comparative studies using extracts from marine sponges have demonstrated that brine shrimp can be a good first indicator of bioactivity, and show similar results to assays tested against fish [52, 56]. Assays were carried out as per methods in [51]. Briefly, a stock solution of the crude extract for each species was prepared by adding a volume of DCM equivalent to that of the extracted tissue. One glass microfiber filter paper (Whatman GF/C 47 mm diam.) was placed into individual glass petri dishes (55 mm diam.) then 0.005, 0.05, 0.5 mL of stock solution were transferred on to the filter papers with a glass pipette. The solvent was left to evaporate from the filter paper under a Nederman arm for 10 min. Brine shrimp eggs were hatched in artificial seawater (Tropic Marin) and twenty actively swimming instar I nauplii (< 12 h after hatching) were collected with a glass pipette and added to each petri dish with 5 mL filtered sea water. Lids were placed on top of the petri dishes and kept under constant illumination for 24 hours. Surviving nauplii (instar

II/III) were then counted; nauplii were considered dead if no movement was detected after several seconds of observation. Natural mortality was controlled for using control treatments in which 0.5 mL of DCM was added to the filter paper. In all cases control deaths occurred, therefore the data was corrected using Abbott's formula  $\% \text{ deaths} = (\text{test} - \text{control}) / (100 - \text{control})$  for analysis [53]. We then calculated the  $\text{LD}_{50}$  of the crude extract for each nudibranch species by interpolating a line or standard curve, chosen based on  $R^2$  values.  $\text{LD}_{50}$  values were calculated for species with extracts that induced a response to at least 50% of the brine shrimp.  $\text{LD}_{50}$  values are interpolated x values (mL stock solution), where 1 mL of extract = 1 mL of tissue, and therefore reflect natural volumetric concentrations. Absolute concentrations of compounds tested are shown in Figures S2 and S3.

#### *Anti-feedant assay*

To assess the relative distastefulness, and thus feeding deterrence of nudibranch extracts, antifeedant assays were performed using the generalist rock-pool prawn (*Palaemon serenus*) between November 2013 and September 2015 as per [51, 54, 55]. This species has a clear carapace and digestive tract, which makes it ideal for feeding observations and preliminary studies have shown that compounds distasteful to marine fish *Tetractenos hamiltoni* and *Rhinecanthus aculeatus* are also distasteful to rock-pool shrimp [56]. Individuals were collected intertidally in SE Queensland on foot using hand nets and housed in aquaria with ample food (Ocean Nutrition, Formula 2) until used in assays. Artificial food pellets were created to approximate the nutritional content of a nudibranch with roughly 90% water, 7% squid + alginate, and 3% sand following the protocol outlined in [51, 57]. Crude extracts were added in several concentrations up to that which they were found occurring naturally for each species by adding the crude stock solution or DCM without extract (control pellets) to a dry mixture (50 mg freeze-dried squid mantle, 30 mg alginic acid, 30 mg purified

sea sand). The DCM of each treatment and control was allowed to evaporate for 30 minutes under a Nederman arm, and then the mixture was reconstituted in distilled water to make a final pellet volume of 0.5 mL. Shrimps were selected randomly and placed individually in small compartments (135mm x 98mm x 90mm) with adequate aeration and water flow. Shrimp were allowed to acclimatize for at least 3 days and fed green fish flakes (Ocean Nutrition, Formula 2) once per day. Shrimp were then starved for 2 days prior to trials. Ten shrimp were randomly selected for each extract-treated and control group. Pellets were offered to shrimp using tweezers and then observed for 60 min. The presence of a red spot in the transparent gastric mill of the shrimp indicated acceptance, and the absence of a spot indicated rejection. Shrimp that rejected a pellet were then offered a control pellet and observed for a further 30 minutes. Shrimp that did not eat control pellets were removed from the analysis. Shrimp were not re-used. The ED<sub>50</sub> of crude extracts was calculated as above.

To consider whether a correlation existed between distastefulness and toxicity while considering phylogenetic relatedness between species, we used a Generalized Least Squares (GLS) regression model. We first pruned the tree to leave only the six species on which we had conducted assays and then created a chronogram using the *chronos* function in the *ape* package v 5.0 [58]. We used the Brownian model [59] as this had the lowest AIC values using *corBrownian*, in comparison to models run with *corGrafen* and *corMartin*. Phylogenetic regression analysis was conducted in R version 3.2.2 [50].

## Results

### *Colour and pattern analysis*

Data for colour and pattern parameters are reported in Table S3 and were visualized in ordinal spacing using NMDS. The red spotted species *Goniobranchus splendidus*, *G. daphne*, *G. hunterae*, *Mexichromis mariei*, *M. festiva* and *Hypselodoris bennetti* formed a

close cluster of similar colour pattern characteristics (Figure 2) from the perspective of a potential predator. *Goniobranchus tasmaniensis* also clustered closely with this group, even though it does not have a yellow rim and spots are orange to human eyes. *Goniobranchus tinctorius* did not cluster close to the main species, presumably due to the presence of a reticulate pattern rather than well-defined spots. Partial red spotted species that did not cluster with the main group were *G. verrieri* and *G. albonares* but neither of these possessed a spotted pattern. Species that were placed in the non-mimic group were widely distributed in the plot. Therefore, our *a priori* groupings based on human vision appeared to be validated, with the exception of the exclusion of *G. tinctorius* and the inclusion of *G. tasmaniensis*, which may reflect differences between human and triggerfish vision.

#### *Phylogenetic relatedness*

The phylogeny generated and stochastic ancestral state reconstruction demonstrates that the red spot group occurs in six parts of the phylogenetic tree (Figure 3) with these included taxa. However, incomplete taxon sampling may affect the reconstruction for some groups, and more conservative estimates might be warranted. However, although the results indicates that shared ancestry may account for similarities in colour pattern for species within the genus *Goniobranchus* and between those in the genus *Mexichromis*, it would not do so between these genera or the other red spot species *Verconia haliclona*, or *Hypselodoris bennetti*. Thus, the red spot pattern has been independently acquired within the family Chromodorididae.

#### *Chemical identification*

Nudibranch species from the red spot mimicry group contained different compounds (Table 1). Species from the genus *Goniobranchus* possessed spongian diterpenes, rearranged

diterpenes, and norditerpenes as per [60], and there were significant differences in chemical profiles between species. Species from *Hypselodoris* and *Mexichromis* species possessed furanosesquiterpenes (Table 1), and the extracts of *M. festiva* from Nelson Bay and the Gold Coast possessed the same compounds. Compound names and structures are listed in Table S4.

#### *Toxicity and palatability assays*

Red spot species differed both in terms of toxicity and distastefulness (Figure 4). Species with extracts that were toxic to brine shrimp included *G. tasmaniensis*, *H. bennetti*, and *M. festiva* (Figure 4A). A dose response was also observed for the extract of *G. daphne*, but this response did not reach above 50% mortality, and no dose response was observed for *G. tinctorius* or *G. splendidus*. All extracts produced a dose response to the shrimp *Palaemon serenus*, though this response did not reach above 50% for the extract of *M. festiva* (Figure 4B). Importantly, using the phylogenetic generalised least square (GLS) regression model, we did not find an association between toxicity and distastefulness ( $t_6 = 0.89$ ,  $p = 0.42$ ; Figure S3).

#### **Discussion**

This study presents quantitative evidence of visual similarities between species in a putative mimicry group using colour and pattern analysis, and demonstrates that shared pattern elements of these co-occurring species are distinct from other, closely related species. Phylogenetic analysis indicates that this red spot pattern evolved at multiple times, suggesting this pattern has resulted from convergent evolution rather than shared ancestry. Members of the mimicry group possess different chemical profiles used for defensive purposes, and these suites of compounds provide unequal levels of defence in terms of a toxic response. However, the level of distastefulness of these compounds appears to be relatively similar to a marine



shrimp. These data therefore do not support the assumption that distasteful compounds honestly signal levels of toxicity, at least in this mimicry system, and in many systems, toxicity may not be related to distastefulness [25]. However, cumulative ingestion could be toxic over time and cause incremental damage or illness. This study should encourage researchers to disentangle terms such as toxicity and distastefulness as modes of chemical defences when investigating aposematic and mimicry systems.

Many theoretical models of mimicry rings with unequal defences exist [e.g. 13, 56-63]. Weakly defended co-mimics may degrade the warning signal of the model [15, 64]; for instance, in an experiment using birds, an increase in abundance of a moderately defended artificial prey increased per capita predation on both the mimic and the highly defended model prey when population densities were low [15]. However, the relationship between species with comparably weak defences and that of their co-mimics remains unclear. In some studies, unequal defences still appear to be mutualistic [14, 65]. For example, highly defended models coupled with moderately defended mimics can have a decrease in per capita mortality when population densities are high [14].

However, the mode of chemical defence is often not defined in such models and unequal defences in mimicry systems are sometimes only discussed in terms of quantity (but see [29]). Prey that store distasteful, but otherwise non-toxic compounds that would not damage or incur costs on the host, may repel predators due to their unpleasant nature. Predators may quickly learn they are not harmed after consuming such prey and may still consume distasteful prey when other food is scarce and predators are hungry [61, 62]. If compounds are equally distasteful, we propose that predators may be unable to discriminate levels of toxicity between species. Therefore, non-toxic species may benefit from resembling their toxic counterparts, but not incur costs involved in harbouring toxins. It is also possible that species may mimic the taste of toxic compounds with those that are non-toxic.

Our study species had very different chemical profiles: *Hypselodoris* and *Mexichromis* nudibranchs contained furanosesquiterpenes while *Goniobranchus* nudibranchs and Chromodorididae *thompsoni* contained spongian diterpenes, nor-diterpenes, and rearranged diterpenes, which appeared to be less toxic than furanosesquiterpenes. Although all chemical extracts in this study were distasteful to *Palaemon* shrimp, this effect was weak for the extract of *M. festiva* (Nelson Bay), which did not induce a response to 50% of the shrimp. *M. festiva* extracts were more concentrated, but contained fewer metabolites than that of *H. bennetti*, which showed enhanced activity in both assays. Therefore, toxicity of these extracts is instead likely to be largely influenced by differences in metabolites. We did not test for an emetic response, which has been shown before in nudibranch compounds [55]. From our results, it appears that chemical defences, both in terms of palatability and of toxicity, are not equal in this mimicry ring. Ideally, toxicity and unpalatability assays would have been conducted on a potential fish predator of nudibranchs, as the response of different taxa to particular compounds may be variable. However, there are considerable ethical implications of conducting toxicity assays with vertebrates.

Our red spot mimetic species clustered together and shared very similar visual characteristics; however, there are some species that shared only some visual similarities and may be considered imperfect mimics. It is predicted that selection on quasi-Batesian mimicry rings should be similar to Batesian systems, with an evolutionary arms race in warning signal design between well-defended and weakly defended species [12, 63]. In this scenario species with greater chemical defences would be selected to differentiate their warning signal from those with weaker defences. However, this hypothesis was not supported in this system, where the colour patterns of the two species with the most potent chemical defences (*G. tasmaniensis* and *H. bennetti*) clustered well with other co-mimics. Alternatively, predators may select for imperfect mimicry in complex Müllerian systems when defended and

palatable prey types are discriminated based on certain components of the visual signal [64], with relaxed selection on other components of the visual signal that are generalized [65]. Indeed, we have recently shown that when learning a red spot / yellow rim colour pattern, triggerfish paid most attention to the yellow border when learning to avoid distasteful food, and disregarded the internal red pattern. We also found that the yellow rim was a more consistent part of the visual signal in populations of *Goniobranchus splendidus*, although there was considerable variation in the red spot component [33]. Highly contrasting body outlines may help nudibranchs to stand out against their background and increase conspicuousness, which is an important characteristic of warning signal designs [5]. However, this does not explain the lack of mantle border in five species in this study.

We believe that this is the first study of an aposematic mimicry ring to include detailed chemical profiles and to assess both the toxicity and distastefulness of contributing species. We have demonstrated that there may not be a correlation between toxicity and distastefulness, and therefore highlight the importance of testing multiple modes of defence to inform future models of mimicry systems. It is likely that warning signal designs and chemical profiles vary geographically [56]; therefore, the impact of geographical differences in dietary resources and predation pressure on warning signal design, chemical profiles, and anti-predator activity of co-mimics would be an interesting direction for future research.

#### **Data accessibility**

Data will be made available through Dryad prior to publication.

#### **Competing interests**

We have no competing interests.

### Author's contributions

AEW participated in fieldwork, lab-work, data analyses, design of the study, and drafted the manuscript; NGW participated the conception of the study, fieldwork, lab-work, data analyses, and drafting the manuscript. CPvdB participated in data analyses, MJH participated in data analyses and drafting the manuscript, JAE participated in data analyses, NJM advised on data analyses, AMW conducted lab-work and identified metabolites. MJG advised on lab-work, assisted with metabolite identification and participated in drafting the manuscript. KLC conceived of, coordinated, and designed the study, participated in fieldwork, lab-work, data analyses, and drafting the manuscript. All authors provided comments on final version and gave approval for publication.

### Acknowledgements

We thank Rachael Templin, Derek Sun, and Will Feeney for help in the field and with spectral reflectance measurements, Holly Urquhart for help with antifeedant assays, and Kara Layton and Diana Prada for assistance with sequencing. This work was supported by The Australian Geographic Society, Experiment.com, The Australia & Pacific Science Foundation (APSF) (to K.L.C, M.J.G. and N.J.M.), UQ Postdoctoral Fellowship (to K.L.C), an Endeavour Postgraduate Award (to A.E.W), the Molecular Systematics Unit at the Western Australian Museum, The University of Queensland, and the Australian Research Council (grants awarded to K.L.C. and N.J.M.).

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## 589 Figure Legends

590 **Figure 1.** Representative photographs of the putative mimicry species investigated in this  
 591 study. **Top panel:** Full pattern including yellow-orange mantle border, white mantle, and  
 592 spots. **Bottom panel:** partial pattern missing either spots or border. From upper left:  
 593 *Goniobranhus splendidus* (A), *Goniobranhus tinctorius* (B), *Goniobranhus daphne* (C),  
 594 *Goniobranhus hunterae* (D), *Mexichromis mariei* (E), *Mexichromis festiva* (F),  
 595 *Hypselodoris bennetti* (G), *Verconia haliclona* (H), *Goniobranhus verrieri* (I),  
 596 *Goniobranhus albonares* (J), *Goniobranhus tasmaniensis* (K), *Chromodorididae thompsoni*  
 597 (L).

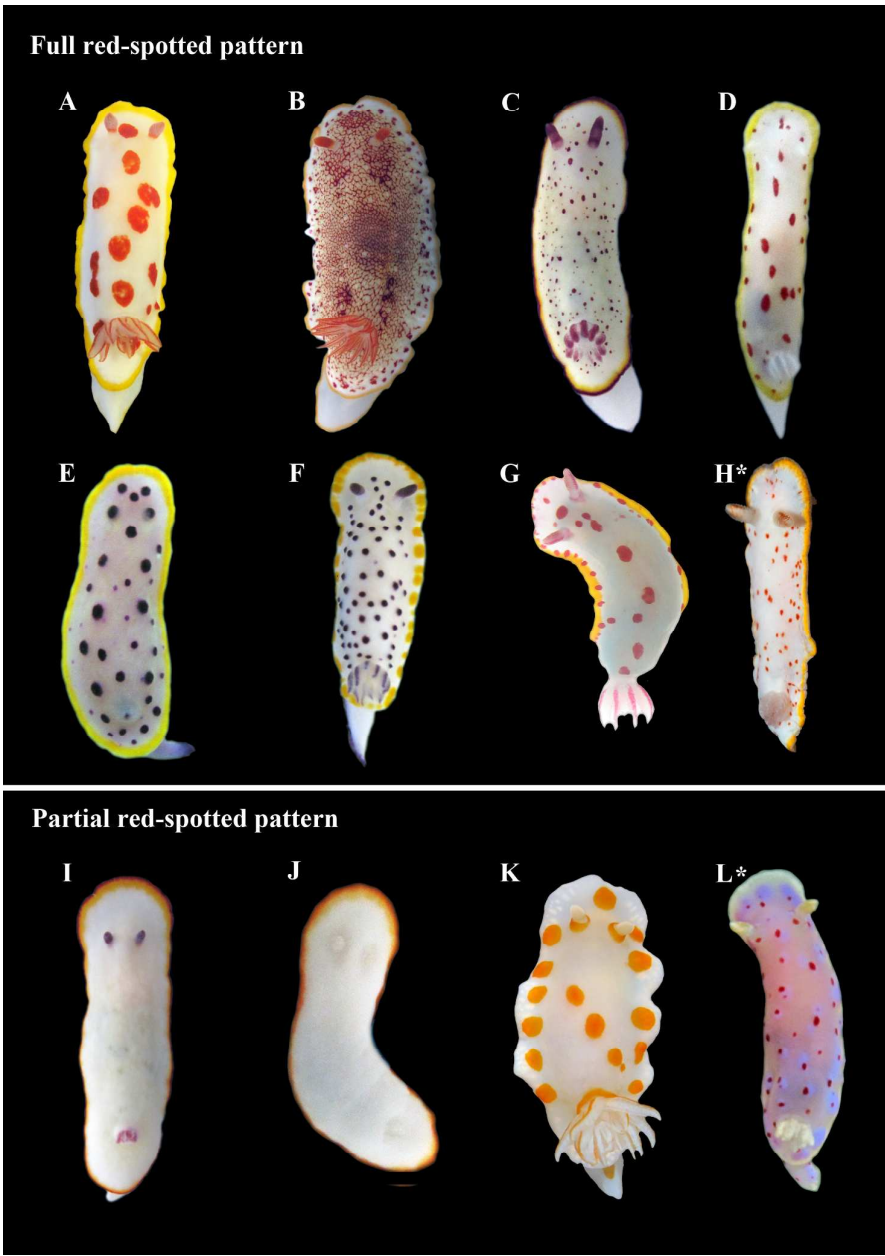
598  
 599 **Figure 2.** Nudibranch colour patterns differentiated in ordinal space (NMDS) based on 14  
 600 metrics of the hue, chroma and luminance of colour pattern element and overall nudibranch  
 601 pattern geometry. The *a priori* predicted red spotted group is shown in red. Partial red  
 602 spotted pattern species are shown in orange and non-red spot group are shown in black. The  
 603 red ellipse shows the clustering of many red spotted species.

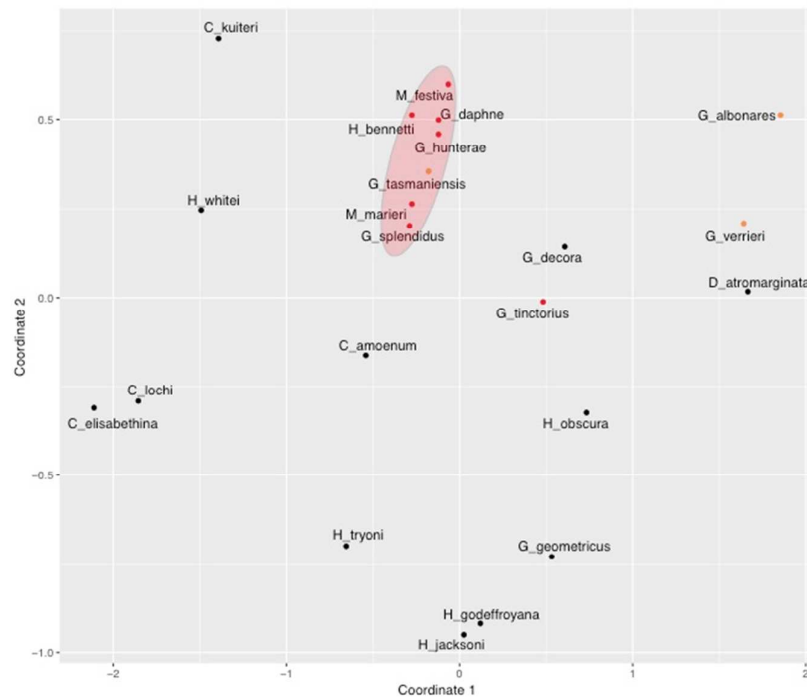
604  
 605 **Figure 3.** Maximum-likelihood topology of Chromodorididae taxa. Species that were  
 606 assigned to a red spotted group are shown in red, the partial red spot group in orange, and  
 607 those not assigned to the non-red spot group in blue. Bootstrap values are shown for clades  
 608 with over 70% support. Ancestral state reconstruction of the red colour pattern was  
 609 performed using ML analysis and marginal probability reconstruction with model Mk1  
 610 (rate 0.24 Log likelihood, -54.77).

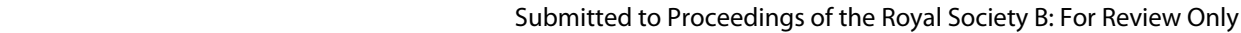
611  
 612  
 613 **Figure 4. a) Toxicity assay:** LD<sub>50</sub> values based on mortality of Brine shrimp, *Artemia* sp. **b)**  
 614 **Anti-feedant assay.** ED<sub>50</sub> values based on rejection of pellets by *Palaemon* shrimp,  
 615 *Palaemon serenus*. Values are represented as proportion of natural concentration found in the  
 616 mantle of the nudibranchs. Circles indicate LD<sub>50</sub> values calculated from the data, nr indicate  
 617 no response at the highest concentration tested. Absolute concentrations are shown in Figure  
 618 S2.

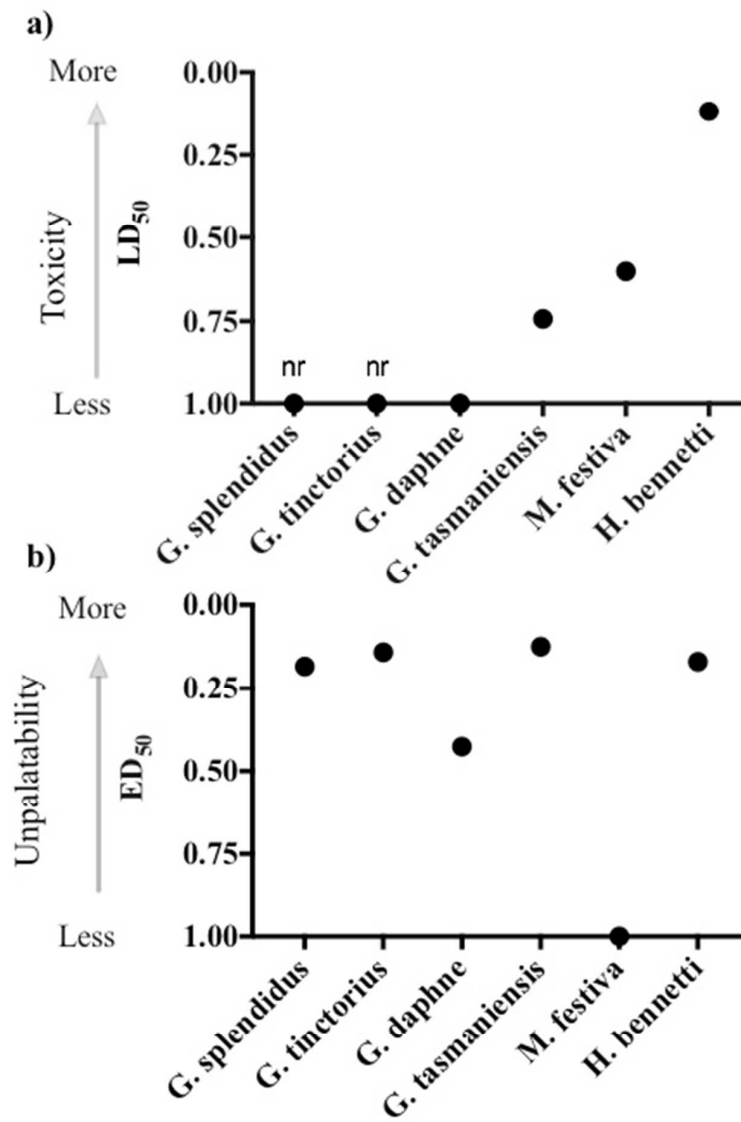
619  
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Figure 1.



**Figure 2.**



**Figure 4.**

**Table 1.**

	Species	Type	Crude mg/ml
Red-spot mimicry species	<i>Goniobranchus splendidus</i>	A, B, C, D	32.4
	<i>Goniobranchus tinctorius</i>	A, B	19.9
	<i>Goniobranchus daphne</i>	B, C	12.3
	<i>Goniobranchus hunterae</i>	B	35.0
	<i>Mexichromis mariei</i>	E	15.3
	<i>Mexichromis festiva</i>	E	17.8 (gcbs) 29.2 (nbps)
	<i>Hypselodoris bennetti</i>	E	15.2
	<i>Veronica haliclona</i>	NA	NA
Partial red-spot species	<i>Goniobranchus verrieri</i>	B, C	19.3
	<i>Goniobranchus albonares</i>	NA	NA
	<i>Goniobranchus tasmaniensis</i>	A, B	37.6
	<i>Chromodorididae thompsoni</i>	B	19.1